Identification of a Poultry Litter Specific Biomarker and Development of a Quantitative Assay

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ACRONYMS

ATCC	American Type Culture Collection
DNA	deoxyribonucleic acid
MDL	method detection limit
MRCF	Molecular Research Core Facility
PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction
T-RF	terminal restriction fragment
T-RFLP	terminal restriction fragment length polymorphism
UV	ultraviolet

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1 INTRODUCTION

This report documents the identification of a potential poultry litter specific biomarker, confirmation of the specificity of that biomarker for poultry litter (as compared to other fecal material), and the development of a quantitative polymerase chain reaction (qPCR) assay specific to detection of the poultry biomarker in various environmental media. Methods and results of the initial tests to identify potential poultry litter specific biomarkers are discussed in Section 2. Testing of the sensitivity of the potential biomarkers toward fecally contaminated litter and soil samples, and the specificity of the potential biomarkers against other fecal samples and various environmental media is presented in Section 3. The development and validation of an assay to quantify the poultry litter specific biomarker in various environmental media is discussed in Section 4.

2 IDENTIFICATION OF POTENTIAL POULTRY LITTER SPECIFIC BIOMARKER

The methods utilized to identify a potential poultry litter specific biomarker are discussed in this section. Specifically, the methods and results of microbial community profiling of poultry litter and soils to which the litter had been applied by terminal restriction fragment length polymorphism (T-RFLP) are discussed in Section 2.1. Cloning bacterial DNA from the litter and soil samples and sequencing of the plasmid deoxyribonucleic acid (DNA) containing the terminal restriction fragments (T-RFs) of interest from these clone libraries are presented in Section 2.2. Comparisons of potential biomarker DNA sequences, as compared to published sequences, and development of polymerase chain reaction (PCR) primers specific to these potential biomarkers are discussed in Section 2.3.

2.1 Litter and Soil Community Profiling

2.1.1 Objective

The purpose of the community profiling through the use of T-RFLP was to generate microbial community profiles of all bacteria, *Escherichia coli*, and *Bacteroides* spp. present in the poultry litter and the soils where poultry litter has been applied. The T-RFLP profiles were then used to identify common microorganisms present in both litter and soils on which poultry litter was spread.

2.1.2 Methods

DNA Extraction. DNA was extracted from two poultry litter samples (five replicates each) and two agricultural soil samples (five replicates each) to which the poultry litter was applied. The poultry litter samples from which DNA was extracted were FAC-01A-1 through 5 and FAC-01B-1 through 5, and the soils samples were LAL3-A-2-1 through 5 and LAL3-B-2-1 through 5. Genomic DNA was extracted with Bio101 DNA extraction kits (QBiogene, Inc).

T-RFLP Analysis. T-RFLP was used to generate community profiles targeting *Bacteria* spp., *E. Coli*, and *Bacteroides* spp. The following steps were used during the T-RFLP analysis.

- The extracted DNA was PCR amplified with phosphoramidite fluorochrome 5-carboxyfluorescein (FAM) labeled universal bacterial primers 8F-907R, with *E.coli* genus specific primers (Tsen, et al. 1998), and *Bacteroides* genus specific primers (Bernhard and Field, 2000). All PCR primers targeted the 16S rRNA gene.

- These PCR products were digested with the restriction enzymes *AciI*, *HhaI*, *MspI*, and *HaeIII* (New England Biolabs).
- Common T-RFs for each PCR primer pair (e.g., universal bacteria, *E.coli* or *Bacteroides spp.*), among all 20 poultry litter and soil replicates, were targeted as potential biomarkers of poultry litter.
- T-RFLP analysis was performed by the Idaho State University, Molecular Research Core Facility (MRCF).

2.1.3 Results

Common T-RFs observed in the soil and litter samples are presented in Table 1. No *Bacteroides* spp. fragments were identified as being present in both the soil and litter samples. T-RFs that were well-represented in all samples were selected for further development.

Table 1. Common T-RFs found in replicate soil and litter samples.

E. coli* PCR products, digested with *MspI				
T-RF[a]	**Litter FAC-01A**	**Litter FAC-01B**	**Soil LAL3-A-2**	**Soil LAL3-B-2**
496.0	1,2,4,5[b,c]	1,2,3,5	1,2,4	Present in all five
498.9	Present in all five[d]	Present in all five	1,2,4,5	Present in all five
500.8	Present in all five	Present in all five	Present in all five	Present in all five
Universal bacteria PCR products, digested with *MspI*				
80.1	1,2,3,4	Present in all five	Not present in any sample	1,3,4
130.9	1,3,4	Present in all five	3	Not present in any sample
142.9	Present in all five	1,2,3,4	1,4	1
147.3	Present in all five	Present in all five	Present in all five	1,4
158.9	2,3,4	Present in all five	2,3,4,5	1,4
165	1,3,4	Present in all five	1,3,4,5	1,4

a: T-RFs of potential biomarkers are indicated in bold typeface.
b: Number indicates the litter or soil replicate sample that the T-RF was identified in.
c: An underlined number indicates that the T-RF represented <1% of community in that replicate.
d: Indicates that this T-RF was detected in all five subsamples within this sample.

2.2 Cloning and Sequencing of Organisms of Interest

2.2.1 Objective

The purpose of the cloning and sequencing was to obtain DNA sequences corresponding to the T-RFs of interest found in both poultry litter and soil upon which poultry litter was applied.

2.2.2 Methods

- Clone libraries were constructed from the original genomic DNA extracted from the soil and litter samples and amplified with either universal bacterial or the *E. coli* genus specific primers. Four universal clone libraries were constructed from the following pooled DNA samples (i.e., 1 µl of genomic DNA extract from each sample was added to the PCR reaction for inclusion into the clones): FAC-01A-1 and FAC-01A-4, FAC-01B-3 and FAC-01B-4, LAL3-A-2-1 and LAL3-A-2-4, and LAL3-B-2-1 alone. Four *E. coli* clone libraries were constructed from the following samples: LAL3-A-2-1, FAC-01B-4, FAC-01A-4, and LAL3-B-2-1. The Topo TA cloning kit (Invitrogen) was used for construction of all clone libraries. DNA samples selected for cloning were from those subsamples in which the T-RFs of interest represented a significant portion of the fluorescence profile.

- The plasmids were excised (QIAprep Spin Miniprep Kit, QIAGEN) from the clones and analyzed by T-RFLP (digested with *MspI* alone) to determine which clones contained the T-RFs of interest (see Table 1).

- Plasmids containing the T-RFs of interested were amplified by PCR and sequenced using the primers T7, T3, 519R, and 338F for double coverage of the 16s rDNA.

2.2.3 Results

- Only three of the six T-RFs representing potential biomarkers were found in the universal clone library (i.e., T-RFs 142.9, 147.3, and 158.9). Sequences representing all three *E. coli* T-RFs were found in the *E. coli* clone library.

- After developing two clone libraries and screening an additional 88 clones with T-RFLP, the target biomarkers with T-RFs of 80.1, 130.6 and 165 were not found. A total of 350 clones were screened.

- T-RFLP sequence analysis and DNA sequencing of each clone was performed by the MRCF.

2.3 Biomarker Sequence Analysis and PCR Primer Design

2.3.1 Objective

The purpose of the biomarker sequence analysis was to compare the potential biomarker DNA sequences to published 16S rRNA DNA sequences to determine whether these sequences had previously been observed, and whether they were identified as a particular species or associated with a particular host animal. Additionally, these sequences were used to design PCR primers specific to each biomarker.

2.3.2 Methods

- The three universal and the three *E. coli* sequences corresponding to the T-RFs of interest were compared to the BLAST database (i.e., National Center for Biotechnology Information) to determine closely related organisms to our potential biomarkers and sites amenable for the design of PCR primers.

- PCR primers were designed for the three biomarkers from the universal bacterial library and one of the *E. coli* biomarkers, targeting regions of variability between our sequence and the database sequences of the top 20 matches in the BLAST database. PCR primers were designed using the Primer Express v2.0 software (Applied Biosystems).These primers were analyzed for thermodynamic folding problems and compared to the RDPII database (Michigan State University) to determine what other organisms they might amplify. The results of the analysis of the forward and reverse PCR primer comparison against the RDPII database are presented in the Table 2.

Table 2. Primer design for potential biomarkers identified during T-RFLP/clone library assessment.

Potential Biomarker Clone Number/ T-RF/Organism *	Forward Primer Accession Number of Closest Match in Sequence Database RPDII	Reverse Primer Accession Number of Closest Match in Sequence Database RPDII
Clone SA19 T-RF 158.9 *Kineococcus*	AY919955, AY426452, AF195447, AF513961, AY862810, AM085954, AM182287, AM182297, AM182298, AM182299	Primer sequence did not match any organisms in the database
Clone SB37 T-RF 142.9 Uncultured organisms	Primer sequence did not match any organisms in the database	Primer sequence did not match any organisms in the database
Clone LA35 T-RF 147.3 *Brevibacterium*	Primer sequence did not match any organisms in the database	Primer sequence did not match any organisms in the database
Clone SA15 T-RF 500.8 *Pantoea ananatis*	Primer sequence did not match any organisms in the database	AJ010486, DQ221344, AF364845, AF364844, AY528223, AY579209, AY579211, U80196, U80209, AB004758, AB027693, AY530796, AJ629190, AB178169, AB178170, AY898643, AB114622, DQ133548, DQ195524, AB242937, AB242945, AB242946, AB242979 And an additional 30 sequences *Enterobacter* spp.
* organsims were identified based on a BLAST search		

2.3.3 Results

- Only one *E. coli* biomarker had a variable region observed in all 20 matches of the closest related organisms in the BLAST database. PCR primers were designed for this variable

region and another region that had a mismatch between our sequence and the database sequence in 10 of the top 20 matching sequences.

- One primer pair was designed that was determined to be specific for T-RF of interest 147.3 from clone LA-35, a *Brevibacterium* spp., and would have no matches compared to the sequences in the RDPII database.

- One primer pair was designed for a *Kineococcus* spp. corresponding to T-RF 158.9, where the forward primer matched the sequences of 10 other organisms in the RDPII database, but the reverse primer was specific to this *Kineococcus* spp.

- One primer pair was designed for an organism not matching any cultured organisms in the BLAST or RDPII databases corresponding to T-RF 142.9.

3 TESTING OF THE SENSITIVITY AND SPECIFICITY OF THE POTENTIAL POULTRY BIOMARKER TARGETS

The methods utilized to test the sensitivity and specificity of the potential poultry litter specific biomarkers are discussed in this section. Specifically, the methods and results for the testing the PCR primers in the original soil and litter samples used for the T-RFLP are presented in Section 3.1, while the testing of the PCR primers for the biomarkers against closely related organisms in the BLAST database search are presented in Section 3.2. Testing of the PCR primers for the various potential biomarkers against other fecal material is presented in Section 3.3. Cloning and sequencing of PCR amplicons derived from fecal samples that amplified with the *Brevibacteria* specific primers and comparison to the biomarker sequence is presented in Section 3.4 Finally, testing of the PCR primers of the confirmed poultry litter specific biomarker in environmental samples from the potentially poultry litter impacted watershed are presented in Section 3.5.

3.1 Test PCR Primers Against Original Soil and Litter Samples

3.1.1 Objective

The objective of this test was to determine if the PCR primers specific for the various biomarkers amplified DNA from the original contaminated soil and poultry litter samples used to find the T-RFs of interest (i.e., potential biomarker sequences).

3.1.2 Methods

- A nested PCR approach was used to increase sensitivity of the PCR assay by first amplifying the DNA with the universal bacterial primers 8F-907R or *E. coli* species-specific primers, purifying the PCR products (QIAquick PCR purification kits, QIAGEN), and then amplifying the DNA with the biomarker-specific primers.

- PCR products were separated by gel electrophoresis, stained with ethidium bromide, and visualized by ultraviolet (UV) light.

3.1.3 Results

The results of the nested PCR with the potential biomarker-specific PCR primers of DNA from the original litter and soil samples are presented in Table 3.

Table 3. Test of the biomarker-specific PCR analysis on original litter and soil replicates.

	Clone LA35 *Brevibacterium* spp.		Clone SB37 Unknown genus		Clone SA19 *Kineococcus* spp.		Clone SA15 *E. coli*	
Sample	% of T-RF Profile	Amplified with LA35 Primers?	% of T-RF Profile	Amplified with SB37 Primers?	% of T-RF Profile	Amplified with SA19 Primers?	% of T-RF Profile	Amplified with SA15 Primers?
Litter Sample Results								
FAC-01-A-1	2.6	yes	2.8	yes	not present[b]	not run	26.4	yes
FAC-01-A-2	2.6	yes	4.6	no	0.8	yes	13	no
FAC-01-A-3	3.4	yes	5.4	yes	1	not run[c]	no data[a]	yes
FAC-01-A-4	3.3	yes	5.9	yes	1.3	not run[c]	23.3	no
FAC-01-A-5	no data[a]	yes	no data[a]	not run[c]	no data[a]	not run[c]	28.5	yes
FAC-01-B-1	3.3	yes	3.6	not run[c]	1.5	not run[c]	33	yes
FAC-01-B-2	4	yes	5.5	yes	1.1	no	43.8	yes
FAC-01-B-3	3.5	yes	6.8	yes	1.3	yes	16.5	yes
FAC-01-B-4	3.2	yes	5.7	not run[c]	1.2	not run[c]	29.1	no
FAC-01-B-5	4.5	yes	8	not run[c]	1.1	not run[c]	48.5	no
Soil Sample Results								
LAL3-A-2-1	7.1	yes	0.8	yes	2.3	not run[c]	6.7	yes
LAL3-A-2-2	12.7	no	not present[b]	yes	3.4	not run[c]	14.3	no
LAL3-A-2-3	9	yes	1	yes	3.6	not run[c]	25.3	yes
LAL3-A-2-4	6.9	yes	0.8	yes	3.2	yes	10.4	yes
LAL3-A-2-5	9.5	no	0.9	yes	3.6	not run[c]	2.5	yes
LAL3-B-2-1	6	yes	0.8	yes	3	yes	18.1	yes
LAL3-B-2-2	no data[a]	yes	no data[a]	yes	no data[a]	yes	6.8	yes
LAL3-B-2-3	not present[b]	yes	not present[b]	yes	3	no	9.1	yes
LAL3-B-2-4	6.3	yes	0.8	yes	3.5	no	2.6	yes
LAL3-B-2-5	no data[a]	yes	no data[a]	yes	no data[a]	yes	7.1	yes

	Clone LA35 *Brevibacterium* spp.		Clone SB37 Unknown genus		Clone SA19 *Kineococcus* spp.		Clone SA15 *E. coli*	
Sample	% of T-RF Profile	Amplified with LA35 Primers?	% of T-RF Profile	Amplified with SB37 Primers?	% of T-RF Profile	Amplified with SA19 Primers?	% of T-RF Profile	Amplified with SA15 Primers?

a ***No data*** indicates that the T-RFLP analysis was not completed on this sample.
b ***Not present*** indicates that the organism represented by that T-RF was not found in the original analysis (see Section 2.1).
c ***Not run*** indicates that this sample was not run with PCR.

3.2 Test PCR Primer Set LA35 Against a Closely Related Bacterium

3.2.1 Objective

The objective of this test was to determine if the PCR primers for the *Brevibacterium* LA35 potential poultry litter biomarker amplified the same product in *Brevibacterium* sp. CHNDP32 (DQ337537), the fourth closest related organism found in the BLAST search.

3.2.2 Methods

- 16S rDNA of the closely related organism identified in the BLAST search was obtained from Dr. Chee-Sanford from the University of Illinois at Urbana-Champaign.
- The *Brevibacterium* sp. CHNDP32 was PCR amplified using the LA35 primers, and PCR products were separated by gel electrophoresis, stained with ethidium bromide, and visualized by UV light.

3.2.3 Results

The *Brevibacterium* biomarkers primers did not amplify the *Brevibacterium* sp. CHNDP32.

3.3 Test PCR Primers Against Other Fecal Samples from Within and Outside the Watershed

3.3.1 Objective

The purpose of this test was to determine the specificity of the four potential poultry biomarker targets (LA35, SB37, SA19 and SA15) against other sources of fecal contamination from within and outside the potentially poultry impacted watershed.

3.3.2 Methods

- Fecal samples were collected in duplicate from beef and dairy cattle, swine, geese, ducks, and humans from inside and outside the potentially poultry impacted watershed. Field blank controls were included with each type of fecal sample. The fecal samples were preserved in glycerol and shipped on ice to the laboratory.
- Genomic DNA was extracted from all fecal samples using the Bio 101 FastDNA SPIN Kit for Soil, DNA extraction kit (Qbiogene, Inc).

- Samples were tested for PCR amplification with the four potential biomarkers, and products were separated by gel electrophoresis, stained with ethidium bromide, and visualized by UV light. Samples were run with a nested PCR approach.

3.3.3 Results

The results of the PCR amplification of the fecal samples with the four potential poultry litter biomarkers are presented in Table 4.

Table 4. Results of the PCR amplification of the fecal samples with the four potential poultry litter biomarkers.

Sample	Type of Fecal Sample	Inside or Outside the Watershed	City	Did the Potential Biomarker Sequence PCR Amplify in the Fecal Sample?			
				Clone LA35 *Brevibacterium*	Clone SB37 Unknown Organism	Clone SA19 *Kineococcus*	Clone SA15 *E. coli*
MAN-BC-1-a	Beef Cattle	Outside		No	No	No	No
MAN-BC-1-b		Outside		No	No	No	No
MAN-BC-2-a		Outside		No	Yes	No	No
MAN-BC-2-b		Outside		No	Yes	No	No
MAN-BC-3-a		Outside		No	No	No	No
MAN-BC-3-b		Outside		No	No	No	No
MAN-BC-4-a		Outside		No	No	No	No
MAN-BC-4-b		Outside		No	No	No	No
MAN-BC-5-a		Outside		No	Yes	Yes	No
MAN-BC-5-b		Outside		No	No	No	No
MAN-BC-6-a		Inside		No	Yes, faint[b]	No	No
MAN-BC-6-b		Inside		No	No	No	No
MAN-BC-7-a		Inside		No	Yes	Yes, faint[b]	Yes
MAN-BC-7-b		Inside		No [a]	Yes	Yes	Yes
MAN-BC-8-a		Inside		No	No	Yes	No
MAN-BC-8-b		Inside		No	No	Yes, faint[b]	No
MAN-BC-9-a		Inside		No	No	No	No
MAN-BC-9-b		Inside		No	No	Yes, faint[b]	No
MAN-BC-10-a		Inside		No	Yes, faint[b]	Yes, faint[b]	No
MAN-BC-10-b		Inside		No	Yes	Yes	No
MAN-BC-F-a	Field Blank	Outside		No	Yes	No	No
MAN-DC-1	Dairy Cattle	Outside		No	Yes	Yes, faint[b]	Yes
MAN-DC-2-a		Outside		No	No	No	No
MAN-DC-2-b		Outside		No	No	No	No
MAN-DC-3		Inside		No	Yes	No	No
MAN-DC-3-b		Inside		No	Yes	No	No
MAN-DC-F	Field Blank	Outside		No	No	No	No
MAN-SW-1-a	Swine	Outside		No	Yes	Yes	No
MAN-SW-1-b		Outside		No	Yes	No	No
MAN-SW-2		Inside		No	No	No	No

Table 4. (continued).

Sample	Type of Fecal Sample	Inside or Outside the Watershed	City	Did the Potential Biomarker Sequence PCR Amplify in the Fecal Sample?			
				Clone LA35 *Brevibacterium*	Clone SB37 Unknown Organism	Clone SA19 *Kineococcus*	Clone SA15 *E. coli*
MAN-DK-1-a	Duck	Outside		No	Yes	Yes	Yes
MAN-DK-1-b		Outside		Yes[a]	Yes	Yes	Yes
MAN-DK-2-a		Outside		No	Yes	Yes	Yes
MAN-DK-2-b		Outside		No	Yes	Yes	Yes
MAN-DK-3-a		Inside		No	Yes	Yes	Yes
MAN-DK-3-b		Inside		No	No	Yes	Yes
MAN-DK-4-a		Inside		No	No	No	No
MAN-DK-4-b		Inside		No	No	No	No
MAN-DK-5-a		Inside		No	Yes	No	No
MAN-DK-5-b		Inside		No	No	No	Yes
MAN-DK-F	Field Blank	Inside		No	No	No	No
MAN-GS-1-a	Goose	Outside		Yes, faint[a, b]	Yes	Yes	Yes
MAN-GS-1-b		Outside		No	Yes	Yes, faint[b]	No
MAN-GS-2-a		Outside		No	Yes	No	No
MAN-GS-2-b		Outside		No	No	No	No
MAN-GS-3-a		Inside		No	No	No	No
MAN-GS-3-b		Inside		No	Yes	Yes	Yes
MAN-GS-4-a		Inside		No	Yes	No	No
MAN-GS-4-b		Inside		No	Yes	No	No
MAN-GS-5-a		Outside		No	Yes	Yes	Yes
MAN-GS-5-b		Outside		No	Yes	Yes	Yes
MAN-HM-1	Waste Water Treatment Plant	Outside	Claremore	No	Yes	Yes	Yes
MAN-HM-2		Inside	Siloam Springs	No	Yes	Yes	Yes
MAN-HM-3		Inside	Fayetteville	No	Yes	No	No
MAN-HM-4	Septic System	Outside	Tulsa	No	Yes	Yes	No
MAN-HM-5		Inside	Fayetteville	No	Yes	No	No
MAN-HM-6		Inside	Siloam Springs	No	No	No	No

a: Samples were re-extracted and re-run in duplicate to confirm result.
b: Faint, indicates a very weak band was observed on the gel.

3.4 Sequencing of the Duck and Goose Amplicon Derived from the LA35 Primer Set

3.4.1 Objective

The purpose of the cloning and sequencing of the duck and goose amplicon derived from the LA35 primer set was to determine if these DNA sequences contained variable regions that could be targeted for a more specific set of PCR primers.

3.4.2 Method

- Several clone libraries were constructed from the duck sample MAN-DK-1-b and the goose sample MAN-GS-1-a by PCR amplifying DNA from these fecal samples with the LA35 primer set, with primers 8F-907R and with 8F-1492R, and cloning with the TOPO-TA cloning kit (Invitrogen). The plasmids were excised from these clones (QIAprep Spin Miniprep Kit, QIAGEN).

- Extracted plasmids were DNA sequenced using the PCR primers T7, T3, 8F, 907R, or 1492R (as appropriate) by the MRCF. Sequences were compared to the poultry litter *Brevibacteria* biomarker using BioEdit V.7.0.5.3 to look for variable regions between the DNA sequences.

3.4.3 Results

The regions of the16S rRNA gene of the duck and goose DNA targeted by the poultry litter specific *Brevibacteria* biomarker PCR primers are identical to our biomarker *Brevibacteria* sequence.

After screening 20 clones from each of the two clone libraries developed (a total of 40 clones) from the duck and the goose fecal samples, only one organism was identified as a *Brevibacteria* using the BLAST database. This sequence was identical to the *Brevibacteria* biomarker sequence obtained from the original soil and litter samples within the 1,200 DNA base pairs sequenced.

3.5 Test for the Poultry Litter Specific Biomarker in Environmental Samples from Within the Impacted Watershed

3.5.1 Objective

The purpose of this test was to determine if the *Brevibacteria* biomarker that is specific to poultry litter could be detected in environmental media (poultry litter, soil and water samples) from within a potentially poultry litter impacted watershed.

3.5.2 Methods

- Environmental samples were collected from within the potentially impacted watershed and included chicken and turkey litter samples, soil samples from which the litter had been applied, edge of field runoff water samples from the fields to which the litter had been applied, river water to which the runoff samples drained, and lakes downgradient from the previously collected river samples. Additionally, groundwater samples within the potentially impacted watershed were collected and analyzed.

- Water samples were collected in duplicate 1-L sterile nalgene bottles and shipped on ice to the laboratory, where they were immediately filtered. The filters containing the microorganisms were frozen at -80°C until DNA extraction.

- Genomic DNA was extracted from the filters and soil and litter samples using the Bio 101 FastDNA SPIN Kit for Soil, DNA extraction kit (Qbiogene, Inc).

- Samples were tested for PCR amplification with the LA35 PCR primers, and products were separated by gel electrophoresis, stained with ethidium bromide, and visualized by UV light. Samples were run with a nested PCR approach

3.5.3 Results

The results of the PCR amplification of the environmental samples for the poultry litter biomarker are presented in Table 5.

Table 5. Results of the PCR amplification of the environmental samples for the poultry litter biomarker.

Sample	Type of Sample	Amplified with LA35 PCR Primers?
FAC1-6-20-06	Litter	Yes
FAC2-6-21-06	Litter	Yes
FAC-03-7-6-06	Litter	Yes
FAC-4-7-12-06	Litter	Yes
FAC-5-7-13-06	Litter	Yes
FAC-6-7-20-06	Litter	No[a]
FAC-7-8-3-06	Litter	Yes
FAC-8-8-15-06	Litter	Yes
FAC-9-8-31-06	Litter	Yes
LAL5-A-2-6-13-06	Soil	No
LAL5-C-2-6-12-06	Soil	Yes
LAL-7-A-2-6-20-06	Soil	Yes
LAL-7-B-2-6-20-06	Soil	Yes, faint[b]
LAL-7-C-2-6-19-06	Soil	Yes, faint[b]
LAL10-B-2-6-26-06	Soil	No
LAL10-A-2-6-26-06	Soil	No
LAL10-A-4-6-26-06	Soil	No
LAL8-A-2-6-19-06	Soil	Yes
LAL8-B-2-6-21-06	Soil	No
LAL9-D-2-6-22-06	Soil	No
LAL9-B-2-6-22-06	Soil	Yes
LAL9-A-2-6-22-06	Soil	Yes
LAL7-D-2-6-29-06	Soil	Yes

Table 5. (continued).

Sample	Type of Sample	Amplified with Poultry Litter Specific *Brevibacteria* PCR Primers?
LAL8-D-2-6-20-06	Soil	Yes
LAL11-C-2-6-28-06	Soil	No
LAL11-D-2-6-28-06	Soil	No
LAL11-A-2-6-29-06	Soil	Yes
LAL11-D-2-Q-6-28-06	Soil	Yes
LAL12-A-2-7-6-06	Soil	No
LAL12-A-2-Q-7-6-06	Soil	Yes
LAL12-C-2-7-7-06	Soil	No
LAL12-D-2-7-7-06	Soil	No
LAL 13-A-2-7-6-06	Soil	Yes
LAL 13-C-2-7-7-06	Soil	No
LAL 13-C-2-Q-7-7-06	Soil	Yes
LAL 13-D-2-7-6-06	Soil	No
EOF-1-6-17-06	Water	No
EOF-Q2-6-17-06	Water	No
EOF-Q1-6-17-06	Water	Yes
EOF-SPREAD073B-6-18-06	Water	Yes
EOF-SPREAD023-6-18-06	Water	Yes
EOF-SPREAD044-6-18-06	Water	No
EOF-SPREAD068-6-18-06	Water	Yes

a: This sample contained a high percentage of soils and very little "litter."
b: Faint indicates that a weak band was visible on the gel.

A summary of the presence or absence of the *Brevibacteria* biomarker in all samples analyzed to date is included in Table 6.

Table 6. Summary of *Brevibacteria* specific PCR results in DNA extracted from fecal and environmental samples.

Type of Sample	Number Analyzed	Number Positive	% Containing LA35 sequence	Note
Original soil and litter samples	20	18	90.0	
Fecal Samples	57	2	3.5	LA35 sequences were present in one duck and one goose sample from outside the watershed
Additional litter samples	9	8	88.9	One of the litter samples (FAC-06) was taken from a poultry house without a concrete floor and likely contained a high percentage of soil.
Additional soil samples	27	14	51.9	
Edge of field water samples	7	4	57.1	

4 OPTIMIZATION AND VALIDATION OF A QUANTITIVE ASSAY FOR THE POULTRY LITTER SPECIFIC *BREVIBACTERIA* BIOMARKER

4.1 Develop a Quantitative PCR Assay for the *Brevibacterium* LA35 Biomarker

4.1.1 Objective

The purpose of this work was to develop a qPCR assay for quantification of the LA35 biomarker in various environmental and fecal samples.

4.1.2 Method

- LA35 PCR primers developed in Section 2 and validated for specificity to poultry litter in Section 3 were applied as qPCR primers using SYBR Green chemistry (dye) on a Chromo4 qPCR system (Bio-Rad).
- Plasmids containing the LA35 DNA sequence were used to create a standard curve, to determine a method detection limit (MDL) for positive control plasmids, and to determine the efficiency of the reaction. All standard curves were run in triplicate to verify the reproducibility of assay.
- Detection limits of the assay in environmental samples were determined by spiking soil and water samples with LA35 contained on plasmids. Controls were run to determine the average number of plasmids present in the *E. coli* cells used as plasmid carriers. Additionally, nanopure water and a composite water sample from the watershed were spiked with the *E. coli* containing the biomarker sequence and were filtered according to

the standard filtering methods. Cloning was performed using the Topo TA cloning kit (Invitrogen). Genomic DNA was extracted from the spiked samples using the Bio 101 FastDNA SPIN Kit for Soil, DNA extraction kit (Qbiogenc, Inc). A graphic depicting the MDL protocol is presented in Figure 1.

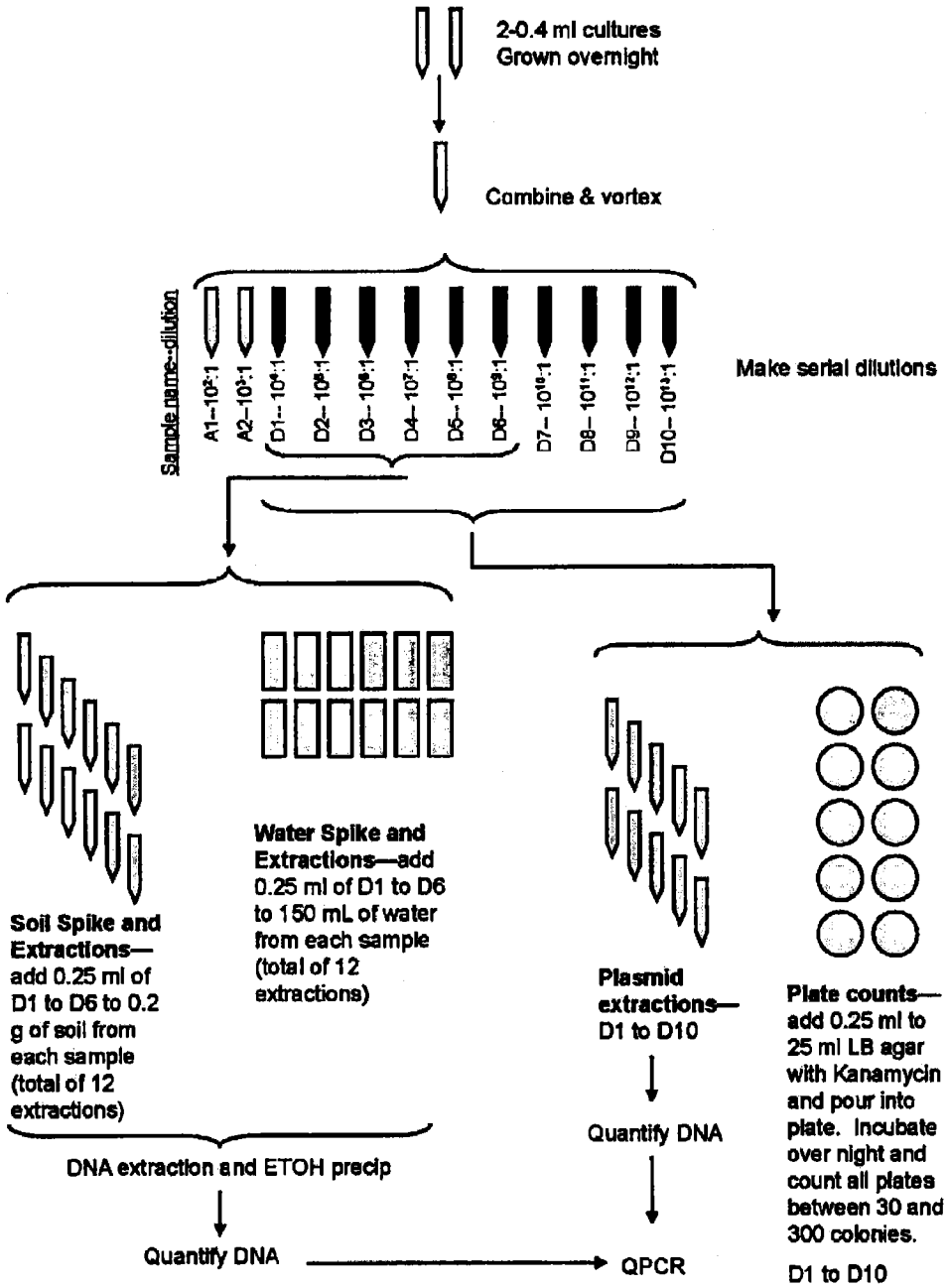

Figure 1. Outline of MDL protocol.

4.1.3 Results

The SYBR green qPCR standard curve is presented in Figure 2. Additionally, the plasmid MDL and reaction efficiency are presented in the figure. Efficiency of the qPCR reaction was determined by equation 1.

Efficiency = -1 + 10 ^ (1-1/slope) (1)

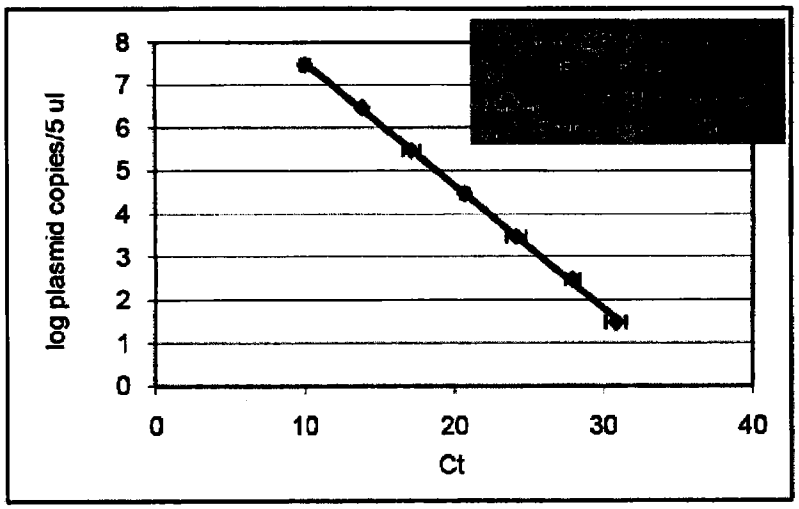

Figure 2. SYBR green qPCR standard curve of the poultry litter specific primers against plasmid DNA containing the *Brevibacteria* biomarker DNA. Error bars indicate the standard deviation of cycle thresholds of triplicate samples.

The results of the minimum detection limit test in spiked environmental samples are presented in Table 7.

Table 7. Detection limit of the qPCR assay for a poultry litter specific *Brevibacteria* in soils and water.

Sample Type	Minimum Detection Limit	Units
Plasmid DNA (standard)	6	copies/µL DNA extraction
Nanopure Water	18	cells/L
Composite Water Sample	78	cells/L
LAL11D-2Q-6-28-06 (soil) with sepharose cleanup	73	cells/g

4.2 Test qPCR Specificity to Distinguish Among *Brevibacteria* spp.

4.2.1 Objective

The purpose of this test was to determine if the qPCR assay is specific enough to distinguish between the poultry litter biomarker and the closely related (but not identical) *Brevibacterium casei* 16S rRNA gene.

4.2.2 Methods

A *Brevibacterium casei* culture was ordered from American Type Culture Collection (ATCC) and the DNA was extracted with the standard protocol. The extracted DNA was then tested for amplification with our qPCR protocol.

4.2.3 Results

Our qPCR primers amplified the *Brevibacteria casei* 16S rRNA gene. As shown in Figure 3, we are able to distinguish *B. casei* from the LA35 sequenceby the SYBR green melt curves.

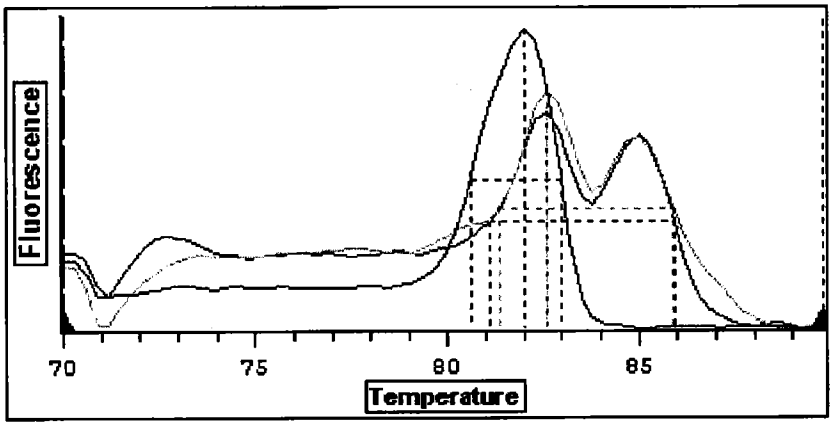

Figure 3. SYBR green melting curve profile of the LA35 16S rRNA sequence (blue) and that of *Brevibacterium casei* [ATCC 35513] (red and green).

4.3 Test for the Poultry Litter Specific Biomarker in Environmental Samples from Within the Impacted Watershed by the qPCR Assay

4.3.1 Objective

The purpose of this test was to determine if the qPCR assay could detect the poultry litter specific biomarker in environmental samples from within the potentially impacted watershed.

4.3.2 Method

- Genomic DNA was extracted from various environmental media (poultry litter, soil and water samples) per the previously described methods. Samples were selected that contained varying levels of fecal indicator bacteria, which was used to gauge the expected biomarker concentration (Table 8)

- DNA was subjected to a diagnostic PCR to verify that the samples did not contain contaminants that might inhibit qPCR and to determine the appropriate sample volume to use for the qPCR assay.

- DNA was then analyzed by the qPCR assay for the poultry litter specific biomarker. Any sample not amplifying in the qPCR assay was tested by a nested qPCR assay, where

universal bacterial primers were first used to amplify the 16S rRNA gene, and then these PCR products were analyzed by the qPCR assay.

4.3.3 Results

The results of the qPCR and nested qPCR assay are presented in Table 8.

Table 8. Results of the test for the LA35 biomarker in environmental samples with the qPCR assay.

Sample ID	Matrix	Expected Biomarker Concentration	DNA (ng/L or ng/g)[a]	qPCR Poultry Specific Biomarker (copies/µL water or g soil or g litter)			qPCR Matrix Spike Amplified?*	Nested qPCR Amplified?*	Biomarker Melt Peak Identified?	Other Melt Peaks Observed?
EOF-spr-010-5-9-06	Water	High	1.7	1.05E+07	±	1.70E+06	Yes	N/A	Yes	No
EOF-spr-17A-01-5-1-06	Water	High	72.5	2.48E+06	±	4.71E+05	Yes	N/A	Yes	Yes
EOF-spr-023-6-18-06	Water	High	4.3	1.11E+05	±	2.49E+03	Yes	N/A	Yes	No
EOF-spread-073B-6-18-06	Water	High	19.2	1.92E+06	±	4.42E+04	Yes	N/A	Yes	No
LAL16-SPR2-7-18-06	Water	High	-1.0	Not present			Yes	No	N/A	N/A
LAL16C-2-7-18-06	Soil	High	9.5	1.42E+04	±	1.97E+03	Yes	N/A	Yes	No
LAL11C-2-6-28-06	Soil	High	73.2	Present, not quantifiable			Yes	Yes	Yes	No
HFS16-BF1-01-6-15-06	Water	Medium	6.8	4.00E+03	±	1.60E+03	Yes	N/A	Yes	No
SALspr-6-28-06	Water	Medium	-0.6	5.82E+02	±	1.56E+02	Yes	N/A	Yes	No
LAL15-SP2-7-11-06	Water	Medium	5.0	2.89E+03	±	7.69E+02	Yes	N/A	Yes	No
RS-PRICEcrk-01-4-29-06	Water	Medium	4.7	3.45E+05	±	1.43E+05	Yes	N/A	Yes	No
RS-574-BIO	Water	Medium	6.7	1.80E+05	±	6.09E+04	Yes	N/A	Yes	No
Lk04-0-01-5-16-06	Water	Low	6.8	3.69E+03	±	3.24E+03	Yes	N/A	Yes	No
HFS28A-BF1-01-6-15-06	Water	Low	-0.7	2.48E+03	±	1.28E+03	Yes	N/A	Yes	Yes
Rs-1-01-8-8-06	Water	Low	7.0	3.19E+04	±	6.75E+03	Yes	N/A	Yes	Yes
FAC-01A-1	Litter	High	33.7	2.18E+09	±	3.53E+08	Yes	N/A	Yes	No
FAC-01A-2	Litter	High	4.7	2.47E+08	±	3.22E+07	Yes	N/A	Yes	No
FAC-01A-3	Litter	High	-0.5	2.67E+07	±	2.69E+06	Yes	N/A	Yes	No

Table 8. (continued).

Sample ID	Matrix	Expected Biomarker Concentration	DNA (ng/L or ng/g)	qPCR Poultry Specific Biomarker (copies/µL water or g soil or g litter)			qPCR Matrix Spike Amplified?*	Nested qPCR Amplified?*	Biomarker Melt Peak Identified?	Other Melt Peaks Observed?
FAC-01A-4	Litter	High	3.4	1.49E+08	±	1.10E+07	Yes	N/A	Yes	No
FAC-01A-5	Litter	High	4.1	5.67E+08	±	3.75E+07	Yes	N/A	Yes	No
FAC-01B-1	Litter	High	94.5	3.94E+09	±	6.28E+08	Yes	N/A	Yes	No
FAC-01B-2	Litter	High	40.5	2.66E+09	±	7.57E+08	Yes	N/A	Yes	No
FAC-01B-3	Litter	High	34.5	4.75E+06	±	4.23E+06	Yes	N/A	Yes	No
FAC-01B-4	Litter	High	117.1	5.99E+09	±	1.74E+09	Yes	N/A	Yes	No
LAL8-A-2-6-19-06	Soil	High	22.34	7.00E+03	±	4.43E+02	Yes	N/A	Yes	No
LAL16B-2-7-18-06	Soil	High	28.94	2.91E+05	±	1.95E+04	Yes	N/A	Yes	No
RS-901-BIO	Water	Low	1.3	Not present			Yes	No	N/A	N/A
LAL16-GW2-7-18-06	Water	None	2.0	Not present			Yes	No	N/A	N/A
CollinsWell#1-7-7-06	Water	None	4.0	Not present			Yes	No	N/A	N/A
66783-7-26-06	Water	None	0.8	Not present			Yes	No	N/A	N/A
LK-01-0-01-8-9-06	Water	None	5.2	Not present			Yes	No	N/A	N/A
Hester-498-8-10-06	Water	None	2.9	Not present			Yes	No	N/A	N/A

N/A, not applicable. The sample was not run with the nested qPCR assay and/or the biomarker melt peak was not identified because none was detected in the qPCR sample run.

Inhibited indicates that the sample did not amplify with qPCR even after a sepharose cleanup was performed and the sample was diluted to a lower DNA concentration.

[a] Concentration in water is given in ng/L; concentraiton in soil is in ng/g

4.4 Determine the Effect of Dilution on Quantification of the LA35 Biomarker in Soil and Water Samples by the qPCR Assay

4.4.1 Objective

This test was conducted to determine a concentration for poultry litter in an environmental sample below which the biomarker is likely not to be detected in environmental samples (i.e., simulate runoff conditions in the lab and test for the presence of biomarker at increasingly dilute samples).

4.4.2 Methods

Litter samples FAC2 and FAC8 (see Table 5) were each carried through the entire procedure. Note that FAC2 was one of the original litter samples used to find LA35. Litter sample FAC8 is a turkey litter sample. Both FAC2 and FAC8 previously tested positive for the poultry litter specific biomarker by the qPCR assay. An outline of the protocol is presented in Figure 4.

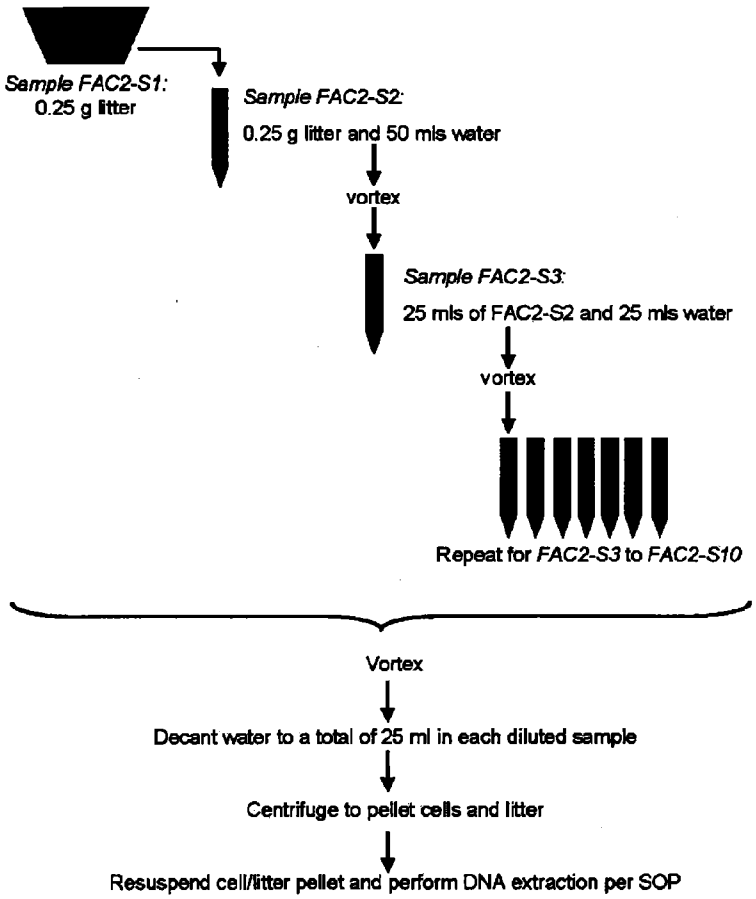

Figure 4. Outline of "dilution limit" protocol using litter sample FAC2 as the example.

4.4.3 Results

LA35 was amplifiable in samples down to 0.1 mg of litter per L. The concentration of LA35 in the litter samples themselves was greater than $2*10^8$ copies/g of litter. There was a very strong correlation between litter concentration (grams of litter per L) and the concentration of LA35 (copies of LA35 per gram of litter, as indicated by the R^2 values of 0.97 and 0.99 shown in Figure 5. These R^2 values indicate that the DNA extraction is efficient and *Brevibacteria* quantification method is relatively precise. Table 9 shows the results if the analysis.

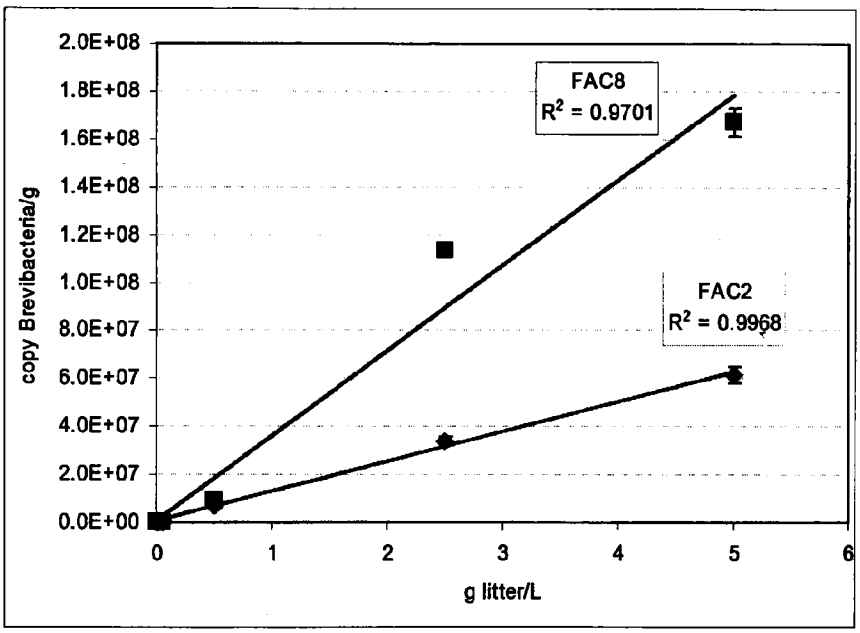

Figure 5. Copies of *Brevibacteria* poultry litter biomarker per gram of soil versus grams of soils per litter in the extractions.

Table 9. Results of the PCR, qPCR, and nested qPCR for *Brevibacteria* in litter.

Sample	"Litter" Concentration^	PCR Bacteria#	Nested qPCR*	*Brevibacteria* 16S rRNA (copy/L water or g litter)
FAC2-S1	NA	+	NA	2.32E+08 ± 8.88E+06
FAC2-S2	5,000 mg/L	+	NA	6.14E+07 ± 3.37E+06
FAC2-S3	2,500 mg/L	+	NA	3.42E+07 ± 1.61E+06
FAC2-S4	500 mg/L	+	NA	6.86E+06 ± 8.27E+05
FAC2-S5	50 mg/L	-	NA	3.54E+04 ± 4.84E+03
FAC2-S6	10 mg/L	-	NA	1.90E+04 ± 4.79E+03
FAC2-S7	5 mg/L	-	NA	1.01E+04 ± 9.35E+03
FAC2-S8	1 mg/L	-	NA	Present, not quantifiable

Table 9. (continued).

Sample	"Litter" Concentration^	PCR Bacteria#	Nested qPCR*	*Brevibacteria* 16S rRNA (copy/L water or g litter)
FAC2-S9	0.1 mg/L	-	+	Present, not quantifiable
FAC2-S10	0.01 mg/L	-	-	Not detected
FAC8-S1	NA	+	NA	2.56E+08 ± 2.49E+07
FAC8-S2	5,000 mg/L	+	NA	1.67E+08 ± 5.88E+06
FAC8-S3	2,500 mg/L	+	NA	1.14E+08 ± 1.56E+05
FAC8-S4	500 mg/L	+	NA	9.05E+06 ± 8.69E+05
FAC8-S5	50 mg/L	-	NA	7.48E+03 ± 2.53E+03
FAC8-S6	10 mg/L	-	NA	7.59E+04 ± 2.97E+04
FAC8-S7	5 mg/L	-	NA	3.20E+04 ± 2.23E+04
FAC8-S8	1 mg/L	-	+	Present, not quantifiable
FAC8-S9	0.1 mg/L	-	-	Not detected
FAC8-S10	0.01 mg/L	-	-	Not detected

^ NA indicates that this was the original 0.25 g undiluted litter sample from which the dilutions were made.

"+" indicates that PCR products were observed after gel electrophoresis, indicating that amplifiable products were obtained from the sample. "-" indicates that PCR products were not observed by gel electrophoresis and UV visualization. Note that the detection limits of the gel electrophoresis method are much higher than the qPCR method detection limits.

* "+" indicates that the *Brevibacteria* were detected with the nested qPCR protocol. "NA" indicates that the samples were not tested with the nested qPCR protocol.

5 REFERENCES

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Tsen, H, et al., 1998, "Development and use of 16S rRNA gene targeted PCR primers for the identification of Escherichia coli cells in water," *Journal of Applied Microbiology*. **85**(3): p. 554-560.